

Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection

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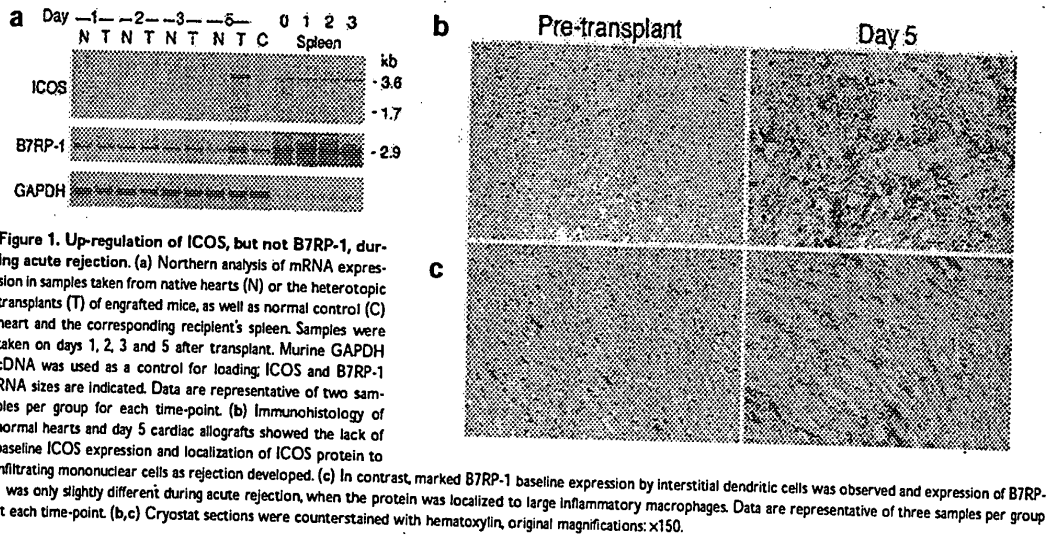
Primary T cell activation requires B7-CD28 and CD40-CD154 costimulation, but effector T cell functions are considered to be largely independent of these costimulatory pathways. Although blockade of costimulation with cytolytic T lymphocyte-associated antigen 4-immunoglobulin (CTLA-4-Ig) or monoclonal antibody (mAb) to CD154 prolongs allograft survival, chronic rejection follows, which suggests that additional key costimulatory pathways are active *in vivo*. We found that both antibody to inducible costimulator (anti-ICOS) and an ICOS-Ig fusion protein suppressed intragraft T cell activation and cytokine expression and prolonged allograft survival in a manner similar to that in ICOS^{-/-} allograft recipients. The combination of anti-ICOS therapy and cyclosporin A led to permanent engraftment. In addition, ICOS-B7RP-1 costimulation was required for the development of chronic rejection after CD40-CD154 blockade. These data demonstrate a key role for the ICOS-B7RP-1 pathway in acute and chronic rejection and highlight the benefits of targeting this pathway in combination with the use of conventional immunosuppressive agent.

The costimulatory molecules CD28 and CD40 ligand (CD154) play key roles in the activation of immune responses after binding of the peptide-major histocompatibility complex (MHC) to the T cell antigen receptor (TCR)¹⁻⁴. Engagement of CD28 by B7-1 (CD80) and B7-2 (CD86) expressed on the surfaces of antigen-presenting cells (APCs) is critical to T cell activation. In addition, cytolytic T lymphocyte-associated antigen 4 (CTLA-4, also known as CD152), a second B7 receptor on T cells which has partial homology to CD28, competes with CD28 for binding to B7-1 and B7-2 on APCs. Blockade of CD28-B7 interactions by administration of the soluble inhibitory fusion protein CTLA-4-immunoglobulin (CTLA-4-Ig) is effective in preventing the progression of disease in experimental models of asthma⁵ and leishmaniasis⁶ and is of therapeutic value in patients with psoriasis⁷. Like the CD28-B7 pathway, ligation of APC-expressed CD40 by CD154 on activated T cells generates key signals for T cell activation, and the use of a monoclonal antibody (mAb) to CD154 is currently being evaluated in several clinical studies. However, although the importance of both CD28 and CD154 in primary T cell activation is well established, these molecules appear to be far less important in the generation and maintenance of memory and effector T cell functions⁸.

Inducible costimulator (ICOS) is the third member of the CD28 family^{9,10}. Unlike CD28, which is constitutively expressed by all T cells, ICOS is expressed preferentially by activated T cells, and studies of ICOS^{-/-} mice show that ICOS costimulation is necessary for the activation and function of effector T cells¹¹⁻¹³. ICOS^{-/-} mice were deficient in interleukin 2 (IL-2) and IL-4 expression, had reduced IL-13 expression^{11,12} and impaired Ig isotype class-switching^{12,13}. The inability to

class-switch was overcome by CD40 stimulation, which suggests that ICOS costimulation is mediated, at least in part, via regulation of the CD40-CD154 pathway¹³. The ICOS ligand was identified as being the third B7 family member and was termed B7 homolog (B7-H)¹⁴; its murine homolog was subsequently cloned and termed B7-related protein 1 (B7RP-1)^{15,16}. B7RP-1 is constitutively expressed by B cells, with lower expression on monocytes.

In transplant models, therapy with CTLA-4-Ig^{17,18} or a CD154 mAb¹⁹, alone or in combination²⁰, prolongs allograft survival, especially when combined with donor-specific therapy (DST) such as intravenous injection of donor spleen cells. However, in the absence of concomitant DST, allografts are eventually rejected due to the development of transplant arteriosclerosis^{21,22}. Given concerns over the clinical applicability of DST, the failure of costimulation blockade alone to prevent chronic rejection has led to a search for additional pathways of potential importance in the regulation of effector T cells. Here we show, using a vascularized cardiac allograft model, that ICOS expression is up-regulated during the development of allograft rejection. The key role of ICOS induction in acute rejection was shown by studies in which therapy with monoclonal anti-ICOS or ICOS-Ig prolonged allograft survival, as well as with the use of ICOS^{-/-} mice. In addition, when used in conjunction with a subtherapeutic regimen of cyclosporin A (CsA), anti-ICOS induced permanent allograft survival without the development of transplant arteriosclerosis. We also show that anti-ICOS therapy prevents chronic rejection after costimulation blockade with a CD154 mAb without the need for DST. Hence, our data show the importance of the ICOS-B7RP-1 pathway in the development of both acute and chronic allograft rejection.



Results

ICOS expression in rejecting heart transplants

We first investigated the extent to which ICOS and B7RP-1 mRNA were expressed during the development of cardiac allograft rejection across a full MHC disparity (from H-2^d to H-2^b). Total RNA was prepared from heterotopic transplants and from each recipient's native heart after collection on days 1–5 after transplant and ICOS and B7RP-1 expression analyzed by northern blot hybridization. Negligible ICOS mRNA expression was present in native hearts, but ICOS was markedly up-regulated by day 5 after transplant (Fig. 1a). Two ICOS-specific mRNAs (3.6 kb and 1.7 kb) were detected in the rejecting transplants. In contrast to ICOS, B7RP-1 (2.9 kb) was readily detectable in normal hearts and mRNA expression did not change markedly during the development of allograft rejection, although a minor increase was observed by day 5 (Fig. 1a). Both ICOS and B7RP-1 were expressed in normal spleen and mRNA levels showed little change after transplant (Fig. 1a).

Given our mRNA data, we sought to localize ICOS protein expression during allograft rejection and determine which cardiac cell types normally expressed B7RP-1. Immunohistological studies with rat mAbs to mouse showed that whereas normal hearts lacked ICOS protein expression, ICOS was localized to infiltrating mononuclear cells, especially by day 5 after transplant (Fig. 1b). In contrast, B7RP-1 was localized to resident interstitial dendritic cells within the normal myocardium and was also expressed during rejection, albeit in low amounts, by infiltrating macrophages (Fig. 1c), consistent with a minor increase in B7RP-1 mRNA expression (Fig. 1a).

ICOS-B7RP-1 and prolonged allograft survival

We examined the role of ICOS up-regulation during acute rejection by comparing the effects of targeting the pathway with a blocking versus a nonblocking mAb to ICOS and determining the effects of an ICOS-Ig fusion protein.

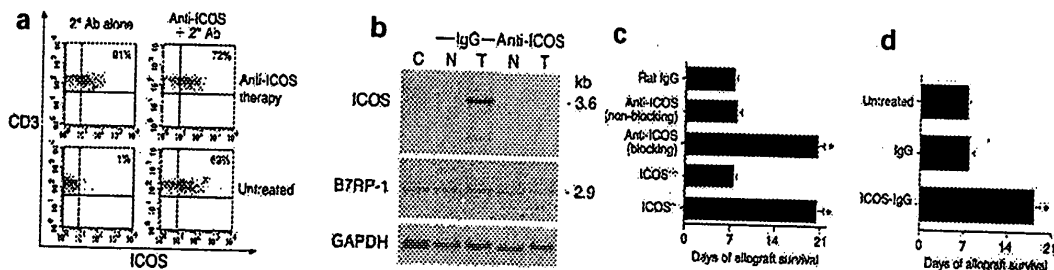
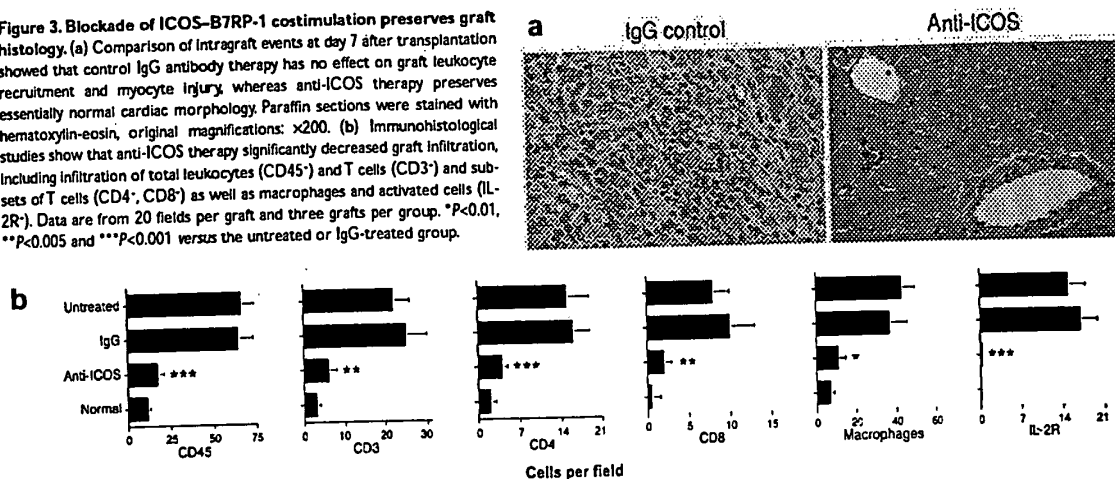


Figure 2. Blockade of ICOS-B7RP-1 costimulation inhibits acute allograft rejection. (a) The anti-ICOS mAb 12A8 did not deplete ICOS⁺ T cells, as shown by flow cytometric analysis of splenocytes 24 h after intraperitoneal injection of mAb (200 µg). Staining of splenocytes with a secondary (2°) antibody alone (mouse anti-rat IgG2b) detected anti-ICOS bound to CD3⁺ splenic T cells in treated, but not control, mice. Additional mAb increased T cell staining only marginally (upper right panel) and untreated mice had similar numbers of ICOS⁺ T cells (lower right panel) as animals receiving anti-ICOS therapy. (b) Northern analysis compared expression of ICOS and B7RP-1 RNA in control hearts versus cardiac allografts collected at day 7 after transplant from recipients treated with IgG or a blocking anti-ICOS (mAb 12A8). A murine GAPDH cDNA fragment was used as a control for loading (data are representative of three grafts per group). Beginning at the time of engraftment, wild-type (ICOS^{+/+}) allograft recipients were treated daily for 14 days with (c) anti-ICOS or (d) ICOS-Ig fusion protein and monitored daily to determine the effects of therapy on allograft survival. Allograft survival in ICOS^{+/+} recipients was also prolonged. Survival data are means ± s.d. and are representative of six animals per group. *P<0.005 compared to other groups, as determined using Mann-Whitney U test.

Figure 3. Blockade of ICOS-B7RP-1 costimulation preserves graft histology. (a) Comparison of intragraft events at day 7 after transplantation showed that control IgG antibody therapy has no effect on graft leukocyte recruitment and myocyte injury, whereas anti-ICOS therapy preserves essentially normal cardiac morphology. Paraffin sections were stained with hematoxylin-eosin, original magnifications: $\times 200$. (b) Immunohistological studies show that anti-ICOS therapy significantly decreased graft infiltration, including infiltration of total leukocytes (CD45⁺) and T cells (CD3⁺) and subsets of T cells (CD4⁺, CD8⁺) as well as macrophages and activated cells (IL-2R⁺). Data are from 20 fields per graft and three grafts per group. * $P < 0.01$, ** $P < 0.005$ and *** $P < 0.001$ versus the untreated or IgG-treated group.



Using flow cytometry, we found that mAb 12A8 (with a specificity for ICOS) bound, but did not deplete, ICOS⁺ T cells *in vivo* (Fig. 2a). This anti-ICOS therapy prevented the up-regulation of ICOS mRNA that is associated with acute rejection at day 7 without affecting cardiac B7RP-1 mRNA expression (Fig. 2b). In addition, although treatment with rat IgG or an isotype-matched nonblocking anti-mouse ICOS had no effect on allograft survival compared with survival in untreated recipients, the

blocking anti-ICOS significantly prolonged allograft survival ($P < 0.005$) (Fig. 2c). The contrasting results obtained with the two types of anti-ICOS indicated that inhibition of ICOS-B7RP-1 interactions is of therapeutic importance in transplantation. This deduction was reinforced by prolonged allograft survival in ICOS^{-/-} recipients that was comparable to that achieved with 12A8 mAb in ICOS^{+/+} mice (Fig. 2c). Therapy with ICOS-Ig, which binds to B7RP-1 and blocks its interaction with ICOS expressed by activated T cells, also prolonged survival ($P < 0.005$) (Fig. 2d).

Histological evaluation of allografts collected at day 7 after transplant showed that therapy with anti-ICOS preserved tissue architecture and blocked host leukocyte accumulation (Fig. 3a). Immunohistological studies showed that anti-ICOS therapy greatly decreased graft infiltration by host T cells, including both CD4⁺ and CD8⁺ T cell subsets, macrophages and activated (IL-2R⁺) cells (Fig. 3b). In contrast, at day 7, natural killer (NK) and B cells were undetectable in any group and only small numbers of neutrophils were detected in areas of myocyte necrosis developing in those recipients receiving IgG therapy or left untreated.

Anti-ICOS suppresses intragraft immune activation

The circulating and intragraft amounts of multiple cytokines, chemokines and their receptors change during transplant rejection²³. We analyzed the expression of cytokines, chemokines and chemokine receptor by RNase protection assays (RPA) to determine the mechanisms responsible for the marked decrease in leukocyte recruitment to allografts of anti-ICOS-treated recipients (Fig. 4). Anti-ICOS therapy suppressed the intragraft up-regulation of cytokine mRNAs, especially interferon- γ (IFN- γ) and IL-10, and reduced the expression of IL-6 and IL-15 mRNAs. Of the chemokines analyzed, lymphotactin (XCL1), RANTES (regulated upon activation, normal T cell-expressed and secreted, or CCL5), eotaxin (CCL11), macrophage inflammatory protein 1 α (MIP-1 α , or CCL3), MIP-1 β (CCL4), MIP-2 (CCL8), interferon- γ -inducible protein-10 (IP-10 or CXCL10), MCP-1 (CCL2) and T cell activation protein 3 (TCA-3 or CCL1) were markedly down-regulated on treatment with anti-ICOS, whereas MCP-1 was not. In addition, anti-ICOS therapy suppressed the

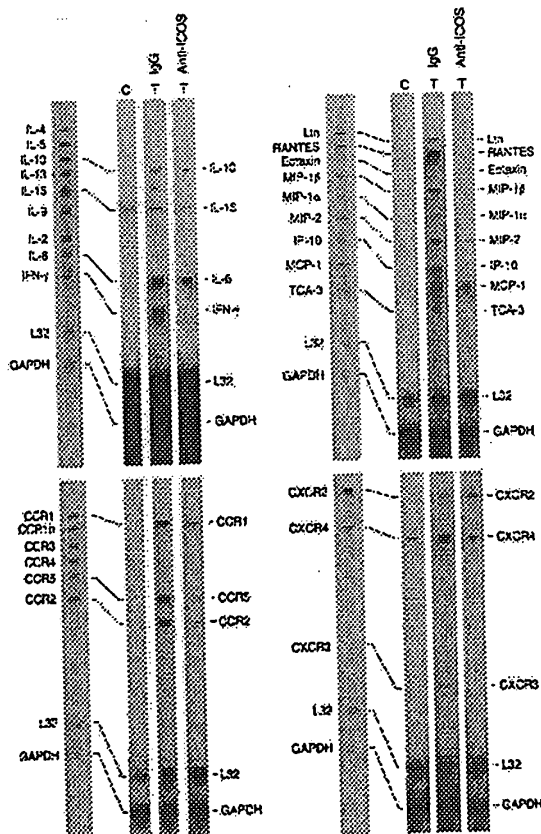


Figure 4. Blockade of ICOS-B7RP-1 costimulation blocks intragraft immune activation. RPAs of intragraft cytokines, chemokines and CC and CXC chemokine receptors were undertaken with the use of total RNA (20 μ g) isolated from control (C) or transplanted (T) hearts at day 7 after transplant; allograft recipients were treated daily with anti-ICOS or an isotype-matched control IgG. Data are representative of three samples per group.

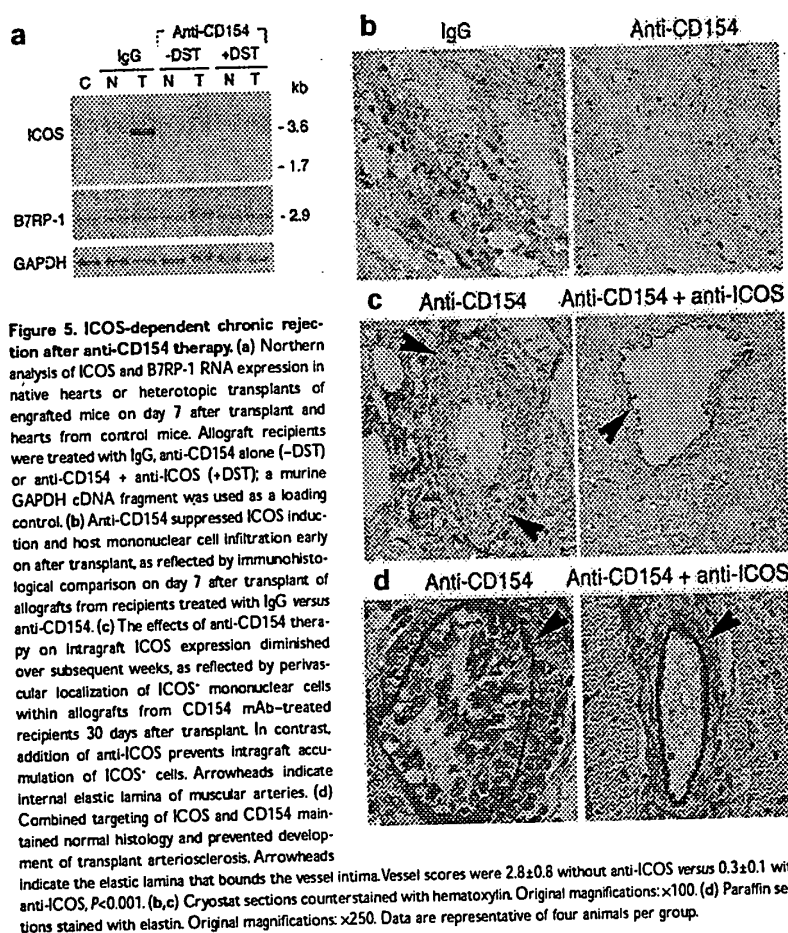


Figure 5. ICOS-dependent chronic rejection after anti-CD154 therapy. (a) Northern analysis of ICOS and B7RP-1 RNA expression in native hearts or heterotopic transplants of engrafted mice on day 7 after transplant and hearts from control mice. Allograft recipients were treated with IgG, anti-CD154 alone (-DST) or anti-CD154 + anti-ICOS (+DST); a murine GAPDH cDNA fragment was used as a loading control. (b) Anti-CD154 suppressed ICOS induction and host mononuclear cell infiltration early on after transplant, as reflected by immunohistological comparison on day 7 after transplant of allografts from recipients treated with IgG versus anti-CD154. (c) The effects of anti-CD154 therapy on intragraft ICOS expression diminished over subsequent weeks, as reflected by perivascular localization of ICOS⁺ mononuclear cells within allografts from CD154 mAb-treated recipients 30 days after transplant. In contrast, addition of anti-ICOS prevents intragraft accumulation of ICOS⁺ cells. Arrowheads indicate internal elastic lamina of muscular arteries. (d) Combined targeting of ICOS and CD154 maintained normal histology and prevented development of transplant arteriosclerosis. Arrowheads indicate the elastic lamina that bounds the vessel intima. Vessel scores were 2.8 ± 0.8 without anti-ICOS versus 0.3 ± 0.1 with anti-ICOS, $P < 0.001$. (b,c) Cryostat sections counterstained with hematoxylin. Original magnifications: $\times 100$. (d) Paraffin sections stained with elastin. Original magnifications: $\times 250$. Data are representative of four animals per group.

up-regulation of mRNAs encoding the chemokine receptors CCR1, CCR2, CCR5, CXCR3 and CXCR4; only the up-regulation of CXCR2 mRNA was unaffected. The importance of this latter observation was unclear because CXCR2 protein is primarily expressed by neutrophils, which were not detected within allografts after ICOS therapy.

Given these data, which show a role for ICOS in the development of acute rejection, involvement of ICOS in the development of chronic rejection after costimulation blockade with anti-CD154 therapy was considered. When administered in conjunction with donor splenocytes as a form of DST, costimulation blockade with CTLA-4-Ig or CD154 mAb can induce permanent survival without the development of chronic

rejection with great effectiveness^{19,24,25}. We therefore tested the effects of CD154 mAb and DST therapy on intragraft ICOS and B7RP-1 expression. Without concomitant DST, allografts in CD154 mAb-treated recipients are rejected due to the development of transplant arteriosclerosis^{21,22}. Therefore we also tested whether targeting of ICOS in conjunction with CD154 mAb only would diminish the incidence and severity of this and other manifestations of chronic rejection.

ICOS up-regulation after CD154 mAb therapy

To determine how ICOS and B7RP-1 RNA levels are affected during anti-CD154 + DST-induced tolerance induction, we compared three groups of allograft recipients by northern analysis at day 7 after transplant. One group received IgG, a second received CD154 mAb alone and a third received anti-CD154 + DST. A fourth group of animals, which were not operated upon or treated in any way, provided normal control cardiac samples. ICOS RNA expression, almost undetectable in the normal heart, was markedly up-regulated during rejection (Fig. 5a). Treatment with anti-CD154, with or without DST, suppressed the intragraft expression of ICOS mRNA completely. However, the baseline level of B7RP-1 mRNA expression was largely unchanged in transplants that were undergoing rejection or tolerance induction (Fig. 5a).

Consistent with the mRNA data, anti-CD154 therapy blocked intragraft ICOS protein expression at day 7, regardless of whether DST was used (Fig. 5b). However, in follow-up studies we found that by 30 days after transplantation, grafts in recipients treated with anti-CD154 developed myocyte and vessel injury in conjunction with prominent intragraft ICOS expression on infiltrating mononuclear cells. In contrast, no ICOS expression or features of chronic rejection were seen in the allografts of recipients treated with CD154 mAb + DST (data not shown). To determine whether the transplant arteriosclerosis that developed after CD154 mAb therapy was ICOS-dependent, we tested the effects of supplementing anti-CD154 with anti-ICOS. We found that, although both protocols extended allograft survival to at least 30

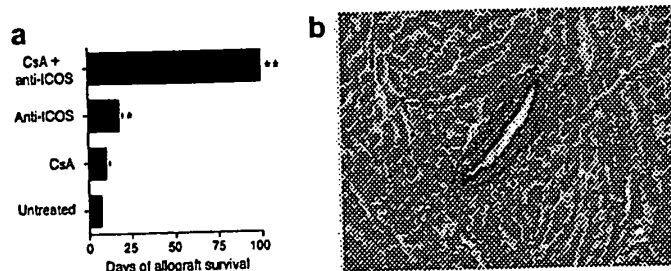


Figure 6. Synergistic effect of CsA therapy and targeting ICOS-B7RP-1. (a) Use of CsA (10 mg/kg/day) for 14 days extended cardiac allograft survival by an extra 3 days. This contrasted with the use of anti-ICOS for 14 days, which tripled cardiac allograft survival ($^{*}P < 0.005$ versus CsA or untreated recipients). However, the combination of CsA + anti-ICOS induced permanent (>100 days) survival ($^{**}P < 0.001$ versus all other groups). (b) Examination, at 100 days after transplant, of long-surviving cardiac allografts from recipients treated with CsA + anti-ICOS showed normal cardiac morphology. Survival and histological data are representative of four animals per group.



days, use of anti-CD154 therapy alone was complicated by the development of florid transplant arteriosclerosis, interstitial fibrosis, focal myocyte necrosis and perivascular accumulation of ICOS⁺ leukocytes (Fig. 5c). In contrast, grafts from animals that had received additional anti-ICOS remained normal, with no intragraft ICOS expression (Fig. 5c) and no evidence of transplant arteriosclerosis ($P < 0.001$) (Fig. 5d).

Synergistic effects of anti-ICOS and CsA

Most current clinical protocols for the management of allograft recipients were developed based upon use of CsA, although it is clear that such protocols do not prevent the development of chronic rejection. We tested whether targeting ICOS might potentiate the effects of CsA, especially with regard to the development of chronic rejection. We found that whereas 2 weeks of therapy with 10 mg/kg/day of CsA (10 mg of drug per kg of body weight per day) prolonged allograft survival by only a few days, combined therapy that used anti-ICOS + CsA for the same period of time led to permanent allograft survival ($P < 0.005$) (Fig. 6a). In addition, examination of the latter allografts at day 100 after transplant showed completely normal cardiac morphology (Fig. 6b).

Discussion

Knowledge of the molecular basis of immune responses is expanding rapidly, driven by a search for key targets within the immune system for control of autoimmune responses and, in the case of transplantation, with an impetus to decrease the incidence of chronic rejection and promote tolerance induction. Recently, much attention in the transplant field has been directed towards costimulation blockade, and encouraging data have arisen from studies to block the CD28-B7 and CD154-CD40 pathways. However, costimulation blockade by either of these approaches usually fails to prevent graft loss because chronic rejection eventually occurs^{22,24,26}. In the case of the CD154-CD40 pathway, which has garnered more overall success in transplant models than CTLA-4-Ig, transplant arteriosclerosis develops regardless of whether anti-CD154 therapy^{22,24} or CD154-deficient recipients²¹ are used. In addition, an anti-CD154 therapy had little effect on the proliferation, differentiation, homing and cytokine production by alloreactive CD8⁺ T cells^{22,24}. This has led to a search for additional costimulatory molecules relevant to the regulation of memory or effector T cell functions.

In this study, ICOS and its ligand, B7RP-1, were implicated in the pathogenesis of acute allograft rejection. We found that ICOS was induced on host mononuclear cells that were progressively infiltrating the graft. A variety of surface molecules are expressed by resting and activated T cells so that use of a depleting antibody directed against any of these membrane proteins could eliminate a key effector cell population, and is therefore sometimes misinterpreted as evidence for the importance of that target in an immune response. However, given contrasting results obtained with a neutralizing *versus* a nonneutralizing mAb to ICOS, data which showed that the neutralizing mAb does not deplete ICOS⁺ cells and the prolongation of allograft survival in ICOS^{-/-} mice, our findings indicate that ICOS induction is important to the development of allograft rejection. Consistent with this, the use of ICOS-Ig also had a beneficial effect on allograft survival, which indicated that blockade of either ICOS itself or the B7RP-1 ligand is of therapeutic value.

The effects of anti-ICOS therapy on allografts include a reduction in the initial T cell and macrophage influx as well as the almost complete abrogation of IL-2R⁺ leukocyte recruitment. These effects are broader than those attributable to the targeting of only ICOS-expressing cells, especially in the case of macrophages that are ICOS⁺, regardless of immune activation. The lack of IL-2R⁺ cells and decreased intragraft expression of the chemokine receptors CXCR3 and CCR5 and the cytokine IFN- γ are con-

sistent with *in vivo* blockade of immune activation. CXCR3 expression, in particular, is induced upon T cell activation and plays a key role in allograft rejection²⁷. CXCR3⁺ T cells infiltrate grafts in response to the initial induction of IP-10 expression by endothelial cells, after ischemia or reperfusion injury, and promote the further up-regulation of IP-10 and other CXCR3 ligands *via* production of IFN- γ ²⁸. IFN- γ is also important to the induction of RANTES and recruitment of CCR5⁺ leukocytes, especially macrophages²⁹. Anti-ICOS therapy did not appear to function *via* immune deviation because the T helper type 2-associated cytokines IL-4 and IL-10 were not expressed within the graft. These findings complement *in vitro* data showing that ICOS delivers a CD28-independent signal for the production of IFN- γ , IL-4 and IL-10⁹.

The development of transplant arteriosclerosis is a hallmark of chronic rejection, a multi-factorial process which currently limits the long-term efficacy of clinical and experimental allografts³⁰. In addition to expression on primary activated T cells, ICOS is expressed by memory T cells⁹ that, on antigen re-exposure, undergo rapid expansion that is independent of CD28-B7 or CD154-CD40 ligation⁴. Therefore, we considered whether ICOS might be important in the development of transplant arteriosclerosis after CD154-CD40 costimulation blockade. CD28 costimulation promotes ICOS induction, and inhibition of CD28-B7 signaling markedly decreases ICOS expression on murine CD4⁺ T cells *in vitro*³⁰. We have shown here that targeting of CD154 suppressed T cell activation, including the induction of ICOS expression on graft-infiltrating T cells. This leads to the question of whether the effects of CD154-CD40 blockade are at least partly ICOS-dependent. However, the long-term effects of anti-CD154 therapy do not appear to be ICOS-dependent because, in conjunction with the development of transplant arteriosclerosis, ICOS expression by intragraft leukocytes gradually returned. The prevention of transplant-induced arteriosclerosis by anti-ICOS therapy clearly establishes the importance of this pathway in the progression and development of chronic rejection and contrasts with the small effect achieved by targeting CD154 or CD28.

When considering targeting a new pathway, it is necessary to assess its potential effectiveness in combination with current best-practice therapy. Most clinical allograft recipients receive CsA-based immunosuppression drugs, although none of these clinical protocols can prevent the long-term development of chronic rejection. There is the added concern that use of inhibitors of calcineurin, like CsA or FK506, may impair development of tolerance induction, as has been observed experimentally^{24,31,32}. We found that anti-ICOS + CsA had synergistic effects that led to permanent engraftment and a complete absence of deleterious host immune responses or development of chronic rejection. Whether this permanent engraftment reflects the development of an unresponsive or anergic state, or possibly points towards a role for ICOS in tolerance induction, is unknown because no third-party allografts or second donor grafts in long-surviving allograft recipients have been undertaken. Nevertheless, we can already note that, in contrast to CD28 or CD154, ICOS is the first major costimulatory molecule in which the benefits of targeting are not impaired by concomitant routine immunosuppression.

Thus, we have described the importance of ICOS-B7RP-1 in allograft rejection. The expression of this pathway is relevant to the pathogenesis of both acute and chronic rejection. The actions of this pathway appear to mediate the development of chronic rejection after CD154-CD40 blockade. In addition, the advantage of targeting ICOS-B7RP-1 is that this therapy acts in synergy with, rather than antagonistically to, conventional CsA-based immunosuppression and generates permanent survival without any evidence of graft injury. Our findings suggest that regulation of this pathway may be of key importance in promoting successful transplantation and possibly in treating autoimmune diseases.

Methods

Reagents. CsA (Sigma, St. Louis, MO) was dissolved in olive oil before use. Hamster anti-mouse CD40L (mAb MR-1) was from BioExpress (West Lebanon, NH) and control hamster and rat IgG antibodies were from Jackson ImmunoResearch (West Grove, PA). Rat anti-mouse ICOS mAbs were generated in-house; of these, mAb 12A8 (IgG2b) blocks binding of B7RP-1 to mouse ICOS cell transfectants, whereas mAb15F9 (IgG2b) is a non-blocking anti-mouse ICOS mAb.

ELISA studies showed that mAb 12A8 (anti-ICOS), used in all *in vivo* studies unless otherwise specified, has a half-life *in vivo* of ~15 h, is cleared by 72 h and elicits a potent mouse anti-rat IgG response by 14 days (data not shown). To test whether this mAb depletes ICOS⁺ cells, we injected mice intraperitoneally with 12A8 (200 µg) and analyzed ICOS expression by splenic CD3⁺ T cells 24 h later. Splenocytes were isolated by mechanical dissociation and treated with PBS containing 10% goat serum to block nonspecific binding. Some splenocytes samples were stained with biotinylated mouse anti-rat IgG2b (PharMingen, San Francisco, CA) to detect residual bound antibody; others were spiked with 12A8 (10 µg/ml) followed by biotinylated mouse anti-rat IgG2b. All samples were subsequently blocked with rat serum, stained with phycoerythrin-streptavidin + rat-anti-mouse CD3 (PharMingen) and analyzed on a FACScan flow cytometer (PharMingen).

A murine ICOS-human IgG1 fusion protein (ICOS-Ig) was prepared as described¹⁹. Rat anti-mouse B7RP-1 was prepared by gene-gun immunization with murine B7RP-1 cDNA, with initial screening by ELISA. This was followed by flow cytometry analysis of mouse B cells and the comparison of binding with ICOS-Ig. We used the clone 3F8 for immunohistological studies.

Transplantation and treatment protocols. Male 6-week-old BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were from The Jackson Laboratory (Bar Harbor, ME) and were maintained in a specific pathogen-free mouse facility. Heterotopic abdominal cardiac allografting was done with the use of BALB/c donors and C57BL/6 recipients¹⁹. The role of ICOS in acute rejection was assessed by intraperitoneally injecting allograft recipients (six individuals per group) with blocking anti-ICOS, nonblocking anti-ICOS or control IgG (500 µg) on alternate days; an additional group received ICOS-Ig (200 µg/day) with the same protocol. Aspects of ICOS involvement in chronic rejection were studied with the use of two methods. For some mice, a single intraperitoneal (i.p.) injection of CD40L mAb (with or without intravenous injection of 5 × 10⁶ donor spleen cells) was administered at transplantation + up to 14 days administration of blocking anti-ICOS or IgG (with the protocol already described). Other mice received 14 days of CsA therapy (200 µg/day, beginning at transplantation) + blocking anti-ICOS or IgG (with the protocol already described). ICOS⁺ mice used as recipients of BALB/c allografts were crossed onto a C57BL/6 background and had the same phenotypic characteristics as three reported lines of ICOS⁺ mice¹⁹.

ICOS cDNA. A plasmid containing the complete ICOS cDNA was from Incyte Genomics (St. Louis, MO). A 556-bp *EcoRI*-*Bam*HI fragment (*EcoRI* site in the vector) that contained ~45 bp of 5'-untranslated sequences and a large part of the ICOS coding sequence (which corresponded to the first 170 amino acids of ICOS) was subcloned into a Bluescript vector and used as a probe in our Northern blot analysis.

Cloning of B7RP-1 cDNA by RT-PCR. Total RNA was isolated from a mixture of murine heart, liver, kidney and spleen with the use of the acid-guanidine thiocyanate-phenol-chloroform method²⁰. A ProStar RT-PCR system (Stratagene, La Jolla, CA) was used for cDNA generation, and later for B7RP-1 cDNA amplification, with primers: 5'-GACT-GAAGTCGGTCAATGG-3' and 5'-CTTCTGCTTGGCAATGCTAG-3'. The 642-bp B7RP-1 cDNA fragment was gel-purified and cloned into a Bluescript vector for use as a probe in Northern blot analysis.

RNA isolations and northern blot analysis of ICOS and B7RP-1 expression. Total RNA was prepared from each recipient's native heart, spleen and cardiac allograft with the use of the acid-guanidine thiocyanate-phenol-chloroform method²⁰. RNA (25 µg) was loaded onto each lane of 1.2% agarose-formaldehyde gels and a 0.24–9.5-kb RNA ladder (Gibco-BRL, Rockville, MD) was used as a size control. After electrophoresis, the RNA was blotted overnight to a Nytran Supercharge membrane (Schleicher and Schuell, Keene, NH) with 20×SSC and cross-linked to the membrane by UV-irradiation with a Stratilinker (Stratagene). ³²P-labeled probes to ICOS and B7RP-1 were prepared with the Multiprime Labeling System (Amersham Pharmacia Biotech, Piscataway, NJ) and hybridizations were performed at 68 °C in roller bottles with ExpressHyb Solution (Clontech, Palo Alto, CA). For reuse, membranes were deprobed in 0.5% SDS at 95–100 °C for 10 min and exposed to film to assure complete removal of previous hybridization signals.

RPA. mRNA levels for several cytokines (mCK-1), chemokines (mCK-5) and CCR- and CXCR-type chemokine receptors were quantified by RPA, according to the manufacturers instructions (PharMingen). Briefly, RNA samples (20 µg each) were hybridized with complementary [³²P]UTP labeled riboprobes, including the probe for the housekeeping gene GAPDH. After hybridization, samples were digested with RNase A, RNase T1 and proteinase K, separated on denaturing polyacrylamide gels and detected by autoradiography with Kodak MR film.

Immunopathology. Histological evaluation was done with the use of paraffin sections stained with hematoxylin and eosin, to assess overall cellularity and myocardial preserva-

tion, and trichrome and elastin stains, to assess interstitial fibrosis and development of transplant arteriosclerosis. Infiltrating cells were detected by immunoperoxidase staining of cryostat sections with rat anti-mouse to CD45⁺ or IL-2R⁺ cells, T cells, macrophages, neutrophils, NK and B cells (PharMingen)²¹ and quantitative morphometry²². Transplant arteriosclerosis was assessed by scoring elastin-stained arteries (less than ten sections per graft and five grafts per group) as <5% occlusion (0), >5–20% (1), >20–40% (2), >40–60% (3), >60–80% (4), or >80–100% (5)²³.

Genbank accession numbers. A plasmid containing the complete ICOS cDNA (GenBank Accession number NM_017480) was used to create a probe for northern analysis.

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